

Mac
R3T.L2

From: Holleran, Anne
Sent: Tuesday, February 10, 2004 3:19 PM
To: STIC-ILL
Subject: refs. for 08/070,099

Please send copies of the following papers:

1. Allen J. Biol. Chem. (1973) 248: 3660-3669
2. Katz J. Clin. Invest (1974) 53: 1274-1283
3. ~~Grasbeck~~ Lancet (1982) I: 1330-1332

Anne Holleran
AU: 1642
Tel: (571) 272-0833
RM: Remsen, 3A14

mailbox: Remsen, 3C18

Preliminary Communication

RADIOIMMUNOASSAY OF URINARY INTRINSIC FACTOR

A Promising Test for Pernicious Anaemia and Gastric Function

RALPH GRÄSBECK VIRVE WAHLSTEDT
ILKKA KOUVONEN

*Minerva Foundation Institute for Medical Research, Helsinki, and
Laboratory Department, Maria Hospital, Helsinki, Finland*

Summary An antiserum against human intrinsic factor (IF) was used for radioimmunoassay of this antigen. Immunoreactive IF was detected in concentrated urine samples from control subjects but not in most of those from pernicious anaemia patients. The IF concentration in urine was 1/10 000 that of gastric juice. It is assumed to be of gastric origin, like urinary pepsinogen.

INTRODUCTION

NUMEROUS proteins of gastrointestinal origin occur in blood and urine; we searched for Castle's intrinsic factor (IF) in these fluids and found it in urine. We compared the amounts excreted by patients with pernicious anaemia and by control subjects.

SUBJECTS AND METHODS

Subjects

Pernicious anaemia patients.—We selected from our records of Schilling tests and from hospital inpatients 20 subjects whose excretion of radioactive vitamin B₁₂ was initially low and clearly increased after the administration of porcine IF. Those selected from the records were asked to bring to the outpatient clinic urine collected overnight from about 2000 h to 0800 h. Subsequent examination of the patients' records showed that all had had mild to pronounced megaloblastic anaemia and low serum cobalamin concentrations. We also studied a female patient with familial selective vitamin B₁₂ malabsorption (Gräsbeck-Imerslund syndrome), who has been described previously.^{1,2}

Control group.—10 healthy laboratory personnel and 14 unselected hospital inpatients formed the control group. About half the patients had diabetes or kidney disease and were included because their 12–24 h urines had been sent to the laboratory for other assays; the other patients were chosen because they were somewhat younger and in better condition than those whose urine samples had been sent to the laboratory; they were recovering from fractures, myocardial infarction, &c.

Methods

Preparation of antiserum.—Pure human IF³ saturated with cyanocobalamin was injected subcutaneously into a rabbit in five doses (108 µg protein each) given 14 days apart. A booster dose was given 7 weeks after the fifth injection, and the antiserum was drawn 18 weeks after the first injection. We showed that it did not cross-react with haptocorrin (R-protein) by adding salivary haptocorrin to the standard tubes used in the radioimmunoassay; there was no interference. We also carried out gel filtration on 'Sephadex G-200' of the antiserum plus human serum saturated with ⁵⁷Co-cyanocobalamin: no radioactivity appeared in the totally excluded volume. This result shows that the antiserum did not react with serum transcobalamin and haptocorrin.

Assay.—Conclusive results could be obtained only if the urine was concentrated 50 times by means of ultrafiltration through dialysis casing. After many experiments with pure IF and different labels we

decided to use labelled IF prepared by incubating human gastric juice and ⁵⁷Co-cobalamin for 30 min at 20°C and diluting the mixture to give in 20 µl 8 fmol (10·4 pg) ⁵⁷Co-cobalamin bound to IF. These data are expressed in moles since one mole of IF binds one mole cobalamin.⁴ The cobalamin-binding capacity of IF in gastric juice was assayed with coated charcoal, after blocking of haptocorrin with cobinamide.⁵ To 100 µl sample or standard we added 5 µl non-radioactive cobalamin (containing 5 ng=4 pmol), 20 µl ⁵⁷Co-cobalamin-IF (10·4 pg=8 fmol bound cobalamin), and 20 µl antiserum (final dilution 1:943), and the reaction mixture was incubated at 4°C in the dark for 17–24 h. 100 µl serum from an unimmunised rabbit (1:500), 100 µl second antibody (pig anti-rabbit-IgG serum 1:6), and 50 µl polyethylene glycol (2 g/10 ml water) were then added and the mixture was incubated at 20°C in the dark for 2 h then centrifuged for 10 min at 1500 g. The supernatant was aspirated and the radioactivity in the precipitate counted. To determine the extent of non-specific binding blanks for each sample were made by replacing the first antibody with the 0·1 mol/l phosphate buffer, pH 7·4, used to prepare all the reagents in the assay. After background and blank correction the bound radioactivity was calculated as a percentage of the total counts in the assay mixture. For use as a standard, gastric juice was diluted with concentrated urine from a patient with pernicious anaemia; the standard curves obtained did not differ from those produced with standards made in serum or buffer. A typical standard curve is shown in fig. 1. The within-assay variation calculated from the radioactivity counts was 5·2% for 0 pmol IF and 7·2% for 0·3 pmol IF contained in 1 ml serum (n=20 at both levels). The lowest detectable amount of IF in the assay was taken to be 0·07 pmol/l, because the standard curve had a plateau just below this concentration.

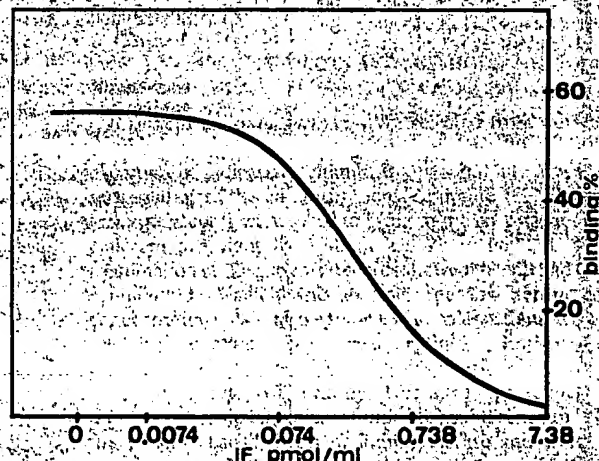


Fig. 1—Typical standard curve.

Standards were made by adding human gastric juice to concentrated urine from a pernicious anaemia patient. IF=cobalamin-binding capacity; % binding=% radioactivity bound to antiserum after background and blank correction.

Urinary IF.—We carried out several experiments to determine the nature of urinary IF (uro-IF). To identify the cobalamin-binding proteins a mixture of concentrated urine and radioactive cyanocobalamin was filtered through sephadex G-200, but no peak with the characteristics of the cobalamin-IF complex was eluted. Because uro-IF was possibly saturated with unlabelled cobalamin we tried to label the IF by incubating the sample at 37°C with ⁵⁷Co-cobalamin and by treating it with guanidine before adding the labelled compound but neither method caused an IF peak to appear after gel filtration. Subsequently three IF-positive urine concentrates were filtered through sephadex G-200 and the eluted fractions assayed for IF by radioimmunoassay.

Other techniques (e.g., purification of IF, gel filtration to detect cobalamin binders) have been used by our group for a long time and have been described elsewhere.^{6,7}

BEST AVAILABLE COPY

EXCRETION OF URO-IF

| Subjects | Age: extreme values (yr) | M/F | Uro-IF in pmol/l urine (n*) |
|-----------------------------|--------------------------|------|--|
| Controls | | | |
| Laboratory personnel | 27-51 | 5/5 | 3 (1) 4 (4) 5 (2) 7 (2) 11 (1) |
| Hospital inpatients | 24-83 | 8/6 | 0+ (2) 2 (5) 3 (1) 4 (5) 5 (1) |
| Pernicious anaemia patients | 53-87 | 5/15 | 0+ (16) 35 (2) 4 (2) |

*n=no. subjects with the observed value.

† Renal insufficiency; serum creatinine over 200 and 300 $\mu\text{mol/l}$, respectively.

‡ 3 resected stomachs, 1 subtotal gastrectomy.

§ In 1 subject repeat 0 pmol/l.

RESULTS

All the controls, except 2 subjects with renal insufficiency, excreted small but significant amounts of uro-IF. 16 of 20 pernicious anaemia patients did not excrete detectable amounts. 4 patients excreted amounts no different from those of the controls, but in 1 of these patients a second urine specimen was negative. In another of the pernicious anaemia patients positive for uro-IF, the activity was shown not to be due to binding-type autoantibodies. The differences between the pernicious anaemia patients and the controls are clear even without statistical tests. The patient with selective vitamin B₁₂ malabsorption excreted 5 pmol/l urine.

Radioimmunoassay of uro-IF-positive urine fractions eluted from sephadex G-200 gave a small activity peak (fig. 2), which emerged somewhat later than the cobalamin-IF complex of gastric juice. In all the fractions the IF concentration was less than that taken as the lowest detectable (0.07 pmol/ml = 59.5% bound), but there was a peak in antiserum-bound radioactivity in fractions 75-80 that

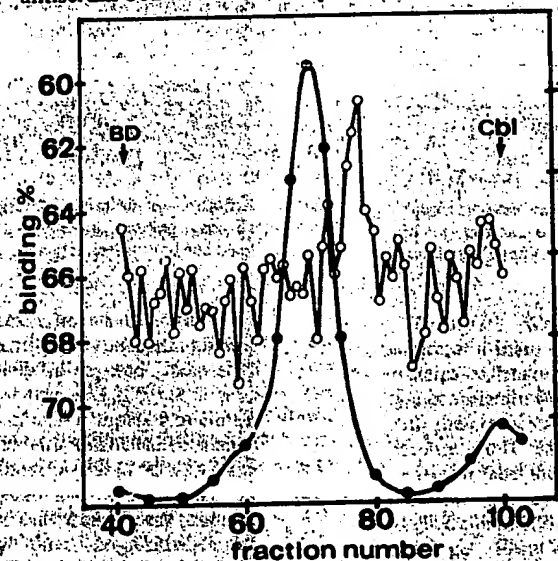


Fig. 2—IF detected by radioimmunoassay in fractions eluted in gel filtration of concentrated urine.

○—○ IF in eluted fractions of concentrated urine.

A similar peak was observed with two other control urines.

●—● ⁵¹Co-cobalamin-labelled gastric juice.

BD=Blue Dextran 2000 (eluted in the totally excluded volume);

Cbl=cobalamin.

seemed to exceed the variation in bound radioactivity in other fractions. There was no such peak in a negative sample from a pernicious anaemia patient. We interpret these findings as indicating that the molecule is not ordinary IF but a smaller one, which perhaps lacks the cobalamin-binding site. We have named it uro-IF in analogy with uropepsinogen.

We confirmed the findings^{8,9} that pepsinogen concentrations in serum and urine are lower than in controls in pernicious anaemia (data not shown). It is possible that uropepsinogen (and the corresponding proteolytic activity) caused the IF activity; however, there was no immunological IF activity in pure solutions of pepsinogen I and no increase in IF activity after pepsinogen was added to our samples.

In preliminary studies we first observed immunological IF activity in serum, but it occurred mainly in the serum of pernicious anaemia patients. Several experiments indicated that this activity was spurious and caused by binding-type autoantibodies against IF. However, the urinary activity was not due to binding-type¹⁰ autoantibodies.

DISCUSSION

We found that urine contains a substance cross-reacting immunologically with IF in gastric juice but not quite identical to it. It was excreted by healthy controls and unselected hospital patients, except those with severe renal insufficiency, but not by most patients with pernicious anaemia. We therefore consider that the assay of uro-IF will prove useful in the routine diagnosis of pernicious anaemia and that it may reduce the need for radioactive-cobalamin absorption tests and assay of the components of gastric juice.

The source of uro-IF could be the intestine where IF is possibly absorbed,¹⁰ but its excretion by a patient with selective cobalamin malabsorption favours our assumption that the source is the gastric mucosa. One drawback of our assay is that the urine has to be concentrated. We hope to improve the sensitivity to eliminate this step and perhaps also to allow detection of IF in serum.

A possible explanation of the unexpected finding of uro-IF in 4 of the 20 pernicious anaemia patients is the difficulty of deciding the level of radioactivity that represents no detectable activity.¹¹ It is possible that such factors and the preliminary nature of our assay protocol may cause false-positive results, but we are inclined to believe that these patients did excrete uro-IF. Our pernicious anaemia patients, except those with resected stomachs, represented what is now regarded as a distinct disease entity—malabsorption of cobalamin due to lack of IF secretion caused by gastric mucosal atrophy. However, mucosal atrophy alone does not seem sufficient to produce cobalamin malabsorption; the presence of autoantibodies against IF appears to be a necessary additional factor.^{10,12} Obviously the mucosal condition passes through different stages, and at one stage antibodies in the mucosa or in the gastrointestinal secretions may inactivate IF.¹² At such a stage the mucosa would still synthesise IF, or a precursor, and this synthesis may be reflected by the presence of uro-IF. It is also possible that the substance excreted is not identical to that found in the controls. It is worth noting that pepsinogen is not always absent either.⁹ Perhaps at some stage of the mucosal degeneration the flow of the products of the gastric glands is prevented, and the glands become endocrine. The serum blocking-type anti-IF titres of the patients excreting uro-IF varied from none to clearly detectable amounts, and their serum pepsinogen I levels were only slightly depressed. Other factors—e.g., the pH and the intestinal bacterial flora—may also help to explain our findings.

BEST AVAILABLE COPY

The absence of uro-IF in 2 patients with severe renal insufficiency is not surprising and hardly reduces the potential usefulness of the test. Because of Bayes' theorem the presence of uro-IF in the urine of some pernicious anaemia patients is a drawback if the test is to be used alone in population screenings.¹³ However, we do not suggest that it should be used alone, rather we envisage its clinical use as one of several tests to elucidate the stage and pathogenesis of gastric mucosal disease and pernicious anaemia and the risk of gastric carcinoma. However, extensive work is needed to improve the sensitivity and to evaluate its usefulness. Such investigations have been initiated.

We thank Prof. Max Siurälä for criticising the manuscript, Dr Frank Laxén for help in examining the patients, Mrs Seija Nordberg for technical assistance, and the Sigrid Jusélius Foundation, the Nordisk Insulinfond, and the Academy of Finland for financial support.

Correspondence should be addressed to R. G. Mielert Institute, P.O. Box 819, SF-00101 Helsinki 10, Finland.

REFERENCES

- Gräsbeck R, Gord n R, Kantero I, Kubbäck B. Selective vitamin B₁₂ malabsorption and proteinuria in young people: A syndrome. *Acta Med Scand* 1960; 167: 289-96.
- Gräsbeck R, Kvist G. La malabsorption congénitale et sélective de la vitamine B₁₂ avec protéinurie. *Cah Col Méd Hôp Paris* 1967; 8: 935-45.
- Allen RH, Mehman CS. Isolation of gastric vitamin B₁₂-binding proteins using affinity chromatography. I. Purification and properties of human intrinsic factor. *J Biol Chem* 1973; 248: 3660-69.
- Gräsbeck R, Simons K, Sinkkonen I. Isolation of intrinsic factor and its probable degradation product, as their vitamin B₁₂ complexes, from human gastric juice. *Biochim Biophys Acta* 1966; 127: 47-58.
- Begley JA, Trachtenberg A, Hall CA. Cobinamide blocking assay for intrinsic factor. In: Zagalak B, Friedrich W, eds. *Vitamin B₁₂. Proceedings of the Third European Symposium on Vitamin B₁₂ and Intrinsic Factor*. Berlin: Gruyter, 1979: 917-18.
- Marcoullis G, Gräsbeck R. Vitamin B₁₂-binding proteins in human gastric mucosa. *Scand J Clin Lab Invest* 1975; 33: 5-11.
- Kouvonen I, Gräsbeck R. A simplified technique to isolate the porcine and human ileal intrinsic factor receptor and studies on their subunit structures. *Biochem Biophys Res Comm* 1979; 88: 358-64.
- Färnsworth EB, Speer E, Alt HL. The quantitative determination of a pepsin-like substance in the urine of normal individuals and of patients with pernicious anaemia. *J Lab Clin Med* 1946; 31: 1025-28.
- Varis K, Samloff IM, Ihamäki T, Siurälä M. An appraisal of tests for severe atrophic gastritis in relatives of patients with pernicious anaemia. *Dig Dis Sci* 1979; 24: 187-91.
- Chanarin I. *The megaloblastic anaemias*. 2nd ed. Oxford: Blackwell, 1979.
- International Atomic Energy Agency, ed. *Radioimmunoassay and related procedures in medicine* 1977. Vienna: IAEA, 1978.
- Toskes PP, Deren JJ. Vitamin B₁₂ absorption and malabsorption. *Gastroenterology* 1973; 65: 662-83.
- Galen RS, Gambino SR. *Beyond normality: the predictive value and efficiency of medical diagnosis*. New York: Wiley, 1975.

Hypothesis

LEWIS BLOOD-TYPE MAY AFFECT THE INCIDENCE OF GASTROINTESTINAL CANCER

HILARY KOPROWSKI
MAGDELENA BLASZCZYK
ZENON STEPLEWSKI

MANFRED BROCKHAUS
JOHN MAGNANI
VICTOR GINSBURG

Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania, and National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.

AMONG many monoclonal antibodies secreted by hybridomas obtained by fusion of mouse myeloma cells with lymphocytes of mice immunised against colorectal cancer cells, several define tumour-associated antigens.¹ Two of these react with the antigen referred to as gastrointestinal-cancer antigen (GICA) present in cells of adenocarcinoma of colon, stomach, and pancreas, but not in other tumour cells or normal tissue.^{2,3} It is also found in meconium.⁴

GICA is a ganglioside whose carbohydrate structure⁵ is:



Fuca1

It is the major ganglioside in the colorectal-cancer cells used for immunisation. Removal of N-acetyl-neuraminic acid from GICA by neuraminidase abolishes monoclonal antibody binding.^{4,6}

GICA IN BLOOD OF PATIENTS WITH GIC AND ON MEMBRANES OF GIC CELLS

Binding of the two anti-GICA monoclonal antibodies to GIC cells is inhibited in solid-state radioimmunoassay by GIC cell extracts or by medium from GIC cells, cultured in vitro, that shed GICA.⁷ In the same assay, sera of some patients with GIC inhibit binding of monoclonal antibody to target cells.³

We have studied the frequency of GICA in sera of 315 patients with GIC, 89 patients with other malignancies, and 108 healthy subjects and on tumour cells from some of the GIC patients.

Anti-GICA monoclonal antibody at a dilution that results in 50% maximum binding reactivity was mixed in equal volume with the subject's serum. The mixture was incubated for 18 h at 4°C and added to polyvinyl chloride plates coated with 3 mol/l potassium-chloride extracts of GIC as a target antigen, followed by further incubation for 18 h with ¹²⁵I-labelled rabbit IgG anti-mouse F(ab)₂. Greater than 12% inhibition of binding by serum was taken to indicate the presence of GICA in serum.

5-μm sections of fixed and embedded tumour tissue were deparaffinised and pretreated with 0.6% hydrogen peroxide in absolute methanol followed by 10% swine sera in phosphate-buffered saline containing 0.1% bovine serum albumin. The slides were then incubated at room temperature, consecutively for 30 min each, with anti-GICA monoclonal antibody, goat anti-mouse F(ab)₂ antibody, swine anti-goat antibody, and goat peroxidase anti-peroxidase antibody. The slides were treated with 0.06% diaminobenzidine, 0.01% hydrogen peroxide in buffer for 5 min, counterstained with haematoxylin, dehydrated, and mounted.

Sera of 64% of patients with clinically and histologically proven colorectal cancer, 92% of patients with pancreatic cancer, and 72% of patients with gastric cancer inhibited binding of the antibody to GICA-containing cancer cells (see table). By contrast, the sera of 8% of patients with malignancies other than GIC and 2% of healthy subjects inhibited binding. Among the 8 patients with tumours other than GIC whose sera inhibited binding of the antibody, 3 had carcinoma of the liver.⁸

Since glycolipids are not destroyed by fixatives such as formalin or Bouin's solution, GICA can be detected in fixed

PRESENCE OF GICA IN SERA AND TUMOUR TISSUE OF PATIENTS WITH GIC AND IN SERA OF NORMAL SUBJECTS

| Subjects | GICA detected in | | | |
|--------------------|------------------|------------|----------------|------------|
| | Sera | | Tumour tissue | |
| | No. of samples | % positive | No. of samples | % positive |
| Colorectal cancer | 255 | 64 | 53 | 55 |
| Pancreatic cancer | 49 | 92 | 17 | 87 |
| Gastric cancer | 11 | 72 | 13 | 100 |
| Other malignancies | 89 | 8 | | |
| Healthy | 108 | 2 | | |

*See text.

BEST AVAILABLE COPY